

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

DSMZ

Deutsche Sammlung von
Mikroorganismen und
Zellkulturen GmbH



INTERNATIONAL FORM

Roche Diagnostics GmbH

Nonnenwald 2, D-82377 Penzberg

F. Hoffmann-La Roche AG

124 Grenzacherstr., CH-4070 Basel

Hoffmann-La Roche Inc.

340 Kingsland Street, Nutley

NJ 07110-1199, USA

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Identification reference given by the DEPOSITOR: <IGF-1R> HUMAB-Clone 1a	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM ACC2586
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I. above was accompanied by: <input type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable).	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2003-04-10 (Date of the original deposit) ¹ .	
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V. INTERNATIONAL DEPOSITARY AUTHORITY	
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¹ Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

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VIABILITY STATEMENT
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Name: Roche Diagnostics GmbH Nonnenwald 2, D-82377 Penzberg Address: F. Hoffmann-La Roche AG 124 Grenzacherstr., CH-4070 Basel Hoffmann-La Roche Inc. 340 Kingsland Street, Nutley NJ 07110-1199, USA		Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM ACC2586 Date of the deposit or the transfer ¹ : 2003-04-10	
III. VIABILITY STATEMENT			
The viability of the microorganism identified under II above was tested on 2003-04-10 ² . On that date, the said microorganism was (<input checked="" type="checkbox"/>) ³ viable () ³ no longer viable			
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED⁴			
V. INTERNATIONAL DEPOSITARY AUTHORITY			
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³ Mark with a cross the applicable box.

⁴ Fill in if the information has been requested and if the results of the test were negative.

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II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
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II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
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IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED⁴			
V. INTERNATIONAL DEPOSITARY AUTHORITY			
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A Chimeric Humanized Single-Chain Antibody against the Type I Insulin-like Growth Factor (IGF) Receptor Renders Breast Cancer Cells Refractory to the Mitogenic Effects of IGF-I¹

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ABSTRACT

Insulin-like growth factors (IGFs) stimulate breast cancer proliferation, motility, and survival. The type I IGF receptor (IGF1R) mediates the effects of IGF-I. Thus, inhibition of IGF1R activation could inhibit IGF action in breast cancer cells. A single-chain antibody directed against IGF1R (IGF1R scFv-Fc) has been shown to partially inhibit xenograft growth of MCF-7 cells in athymic mice. In this study, we have examined the effects of scFv-Fc on IGF1R signaling in the estrogen receptor-positive (ER+) MCF-7 breast cancer cells *in vitro* and *in vivo*. The antibody stimulated IGF1R activation *in vitro* in MCF-7 cells and was unable to block IGF-I effects. The antibody also stimulated proliferation of MCF-7 cells in monolayer growth assays. To determine how scFv-Fc could stimulate *in vitro* growth yet inhibit *in vivo* tumor growth, we examined the effect of scFv-Fc on IGF1R expression. In MCF-7 cells, scFv-Fc down-regulated IGF1R levels after 2 h, and the levels were greatly reduced after 24 h. In contrast, IGF-I treatment over the same time period did not affect IGF1R levels. Twenty-four-h pretreatment of cells with scFv-Fc blocked IGF-I mediated phosphorylation of insulin receptor substrate-1 and subsequent extracellular signal-regulated kinase 1/extracellular signal-regulated kinase 2 and phosphatidylinositol 3'-kinase activation. In contrast, cells treated with 5 nM IGF-I for 24 h still retained the ability to further activate downstream signaling pathways in response to IGF-I. Moreover, pretreatment of MCF-7 cells with scFv-Fc rendered them refractory to further proliferation induced by additional IGF-I. Twenty-four-h pretreatment of cells with scFv-Fc also inhibited IGF-I stimulated anchorage-independent growth. scFv-Fc did not enhance antibody-dependent cell-mediated cytotoxicity. *In vivo*, treatment of mice bearing MCF-7 xenograft tumors with scFv-Fc resulted in near complete down-regulation of IGF1R. Our data show that scFv-Fc stimulates biochemical activation of IGF1R, then causes receptor down-regulation, making MCF-7 cells refractory to additional IGF-I exposure. These results indicate that such chimeric single-chain antibodies against IGF1R have future potential in breast cancer therapy by causing down-regulation of receptor.

INTRODUCTION

Some breast cancer cells proliferate in response to the IGFs.³ In other cells, IGFs enhance motility and survival (1, 2). These effects are mediated by the IGF1R, which consists of two covalently linked polypeptide chains, each with an extracellular α -subunit and a trans-

membrane β -subunit that contains tyrosine kinase activity. The IGF1R is transported to the membrane fully assembled in the dimeric form and after ligand binding, tyrosine kinase activity is initiated. Activation of this receptor protects breast cancer cells from chemotherapy (3, 4), causes radioresistance (5), and may be required for oncogenic transformation and tumorigenicity of cells (6). Since the kinase activity of IGF1R has been reported to be enhanced in breast cancer (7), it may be a potential target for therapy (8). The success of trastuzumab, a humanized antibody against HER2/c-erbB2 (9, 10), in the treatment of breast cancer patients (11) and the small molecule tyrosine kinase inhibitor imatinib mesylate (STI-571) that blocks the kinase activity of the Bcr-Abl oncoprotein and c-kit tyrosine kinase in the treatment of chronic myelogenous leukemia (12, 13) and gastrointestinal stromal tumors (14) have proved that antitumor factor receptor and tyrosine kinase inhibition therapy have important clinical benefits.

IGF1R has been reported to be important in several different cancers, including breast cancer, prostate cancer, liver cancer, glioblastomas, and childhood malignancies, and several approaches have been used to inhibit signaling via IGF1R. An antisense IGF1R oligodeoxynucleotide-based therapy is currently in clinical trial in patients with astrocytomas (15). A second way to inhibit IGF1R is the use of small molecule inhibitors that would inhibit the tyrosine kinase activity by binding to the ATP binding site or substrate binding site in the kinase domain of IGF1R or by blocking substrate binding to the activated receptor. Several small molecule inhibitors of IGF1R are currently being studied, but to date, there are no reported inhibitors specific for IGF1R (16, 17). Unlike other growth factor receptor tyrosine kinases, IGF1R is only activated by ligands; overexpression alone does not lead to its activation as is seen in the case of Her2/neu (18, 19). Therefore, any approaches that prevent binding of ligands to IGF1R may inhibit activation of the receptor.

Several antibodies have been studied as a strategy to inhibit IGF1R activation. Among these, the monoclonal antibody α IR3 (20), which blocks binding of IGF-I to IGF1R, has been widely studied. α IR3 inhibits proliferation of MCF-7 cells *in vitro* (21, 22). It also inhibits the growth of some breast cancer cell lines such as MDA-MB-231 and T61 *in vivo* (23) but not the xenograft growth of IGF responsive cells such as MCF-7 (24).

In this study, we have used a single-chain antibody that inhibits the binding of IGF-I and IGF-II to IGF1R (25) to determine its effect on IGF1R signaling. The anti-IGF1R scFv-Fc (referred to as scFv-Fc) used in this study was engineered from a mouse monoclonal antibody, 1H7, that blocks binding of IGF-I and IGF-II to IGF1R (26). scFv-Fc is a chimeric antibody that has the Fc domain of human IgG1 fused to the Fv region of the mouse monoclonal antibody 1H7. scFv-Fc has been shown to partially inhibit the xenograft growth of MCF-7 cells (25).

In MCF-7 cells, we have shown that IGF-I activates IGF1R, which phosphorylates the adaptor protein IRS-1 (27). IRS-1 then recruits other signaling molecules, resulting in the activation of further downstream pathways including the MAPK and PI3K pathways. The ob-

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³ The abbreviations used are: IGF, insulin-like growth factor; IGF1R, type I IGF receptor; IRS-1, insulin receptor substrate-1; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3'-kinase; HRP, horseradish peroxidase; ERK, extracellular signal-regulated kinase; IGF1R β , β -subunit of IGF1R; IGFBP-1, IGF-binding protein 1; IMEM, Improved Minimum Essential Medium; SFM, serum-free media; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ADCC, antibody-dependent cell-mediated cytotoxicity; NK, natural killer.

jective of this study was to identify the mechanism of action of scFv-Fc on IGF1R function in MCF-7 cells. We found that scFv-Fc acted as a full agonist in MCF-7 cells and activated IGF1R tyrosine kinase activity to stimulate monolayer growth. However, scFv-Fc, but not IGF-I, down-regulated levels of IGF1R over time. Cells exposed to scFv-Fc became refractory to additional IGF stimulation, supporting a role for this antibody as an anti-IGF therapeutic.

MATERIALS AND METHODS

Reagents. All reagents and chemicals were purchased from Sigma (St. Louis, MO), and cell culture reagents were from Invitrogen/Life Technologies, Inc. (Rockville, MD) unless otherwise noted. IGF-I was purchased from GroPep (Adelaide, Australia), and the anti-IGF1R scFv-Fc was engineered and purified as described previously (25). Antiphosphotyrosine antibody (PY-20) conjugated to HRP was from BD Transduction Laboratories (Lexington, KY). Antibodies against ERK1/ERK2 (phosphospecific and total) and Akt (phosphospecific and total) were purchased from Cell Signaling (Beverly, MA). The rabbit polyclonal antibody against IRS-1 was produced by Alpha Diagnostics (San Antonio, TX) by immunizing rabbits with a 14-amino acid peptide (YASINFKPEDRQ) from the COOH-terminal region of IRS-1. The immune serum was affinity purified on immobilized protein A column using the ImmunoPure IgG purification kit (Pierce, Rockford, IL). The polyclonal antibody against IGF1R β was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antirabbit secondary antibody conjugated to HRP was from Amersham Biosciences (Piscataway, NJ). Protein A-agarose was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Acrylamide, bis-acrylamide, and prestained molecular weight markers were from Bio-Rad (Hercules, CA). Recombinant human IGFBP-1 expressed in *Escherichia coli* (28) was originally obtained from Synergen (Boulder, CO).

Cell Lines and Culture. MCF-7 cells were obtained from Dr. C. Kent Osborne (Baylor College of Medicine, Houston, TX) and were routinely maintained in IMEM with Zinc Option (Richter's modification) with 5% fetal bovine serum, 11.25 nM human insulin (Eli Lilly, Indianapolis, IN), 50 units/ml penicillin, and 50 μ g/ml streptomycin. NIH3T3 cells overexpressing IGF1R, NIH3T3/IGF1R (29), were obtained from Dr. Reiner Lammers (Department of Internal Medicine IV, Tübingen, Germany) and grown in DMEM supplemented with 10% fetal bovine serum.

Cell Stimulation. MCF-7 cells were grown in 10-cm dishes in regular growth media. Confluent cells (70%) were washed twice with PBS and serum deprived for 24 h in SFM as described previously (27). For treatment with IGF-I or scFv-Fc, medium was replaced with SFM containing 5 nM IGF-I or 250 nM scFv-Fc for times as indicated in the figure legends. To determine whether scFv-Fc inhibited IGF-I mediated activation of IGF1R or other pathways, cells were first pretreated with scFv-Fc for 30 min or 24 h and then with IGF-I for an additional 10 min. To determine whether down-regulation of IGF1R by scFv-Fc occurs by the proteasome pathway, cells were pretreated with 30 μ M MG115 (Calbiochem, San Diego, CA), a proteasome inhibitor, for 2 h before treatment with IGF-I or scFv-Fc. To determine the effect of a lysomotrophic agent on scFv-Fc mediated down-regulation of IGF1R, cells were pretreated with 40 mM methylamine for 4 h before treatment with IGF-I or scFv-Fc for various times.

Cell Lysis. Cells were washed three times with ice-cold PBS on ice and lysed with 500 μ l/10-cm dish lysis buffer TNESEV [50 mM Tris-Cl (pH 7.4); 1% NP40; 2 mM EDTA (pH 8.0); 100 mM NaCl, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μ g/ml leupeptin, and 20 μ g/ml aprotinin]. Lysates were clarified by centrifugation at 12,000 \times g for 20 min at 4°C, and soluble cellular proteins were used for experiments or stored at -20°C. Protein concentrations of the lysates were determined using the bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL).

Immunoblotting. For immunoblotting, 40 μ g of cellular proteins were subjected to reducing SDS-PAGE on 8% polyacrylamide gels after the Laemmli system (30). After SDS-PAGE, proteins were transferred to nitrocellulose. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with Tween 20 [TBST - 0.15 M NaCl; 0.01 M Tris-HCl (pH 7.4); 0.05% Tween 20] for 1 h at room temperature with gentle shaking. Membranes were then washed five times with TBST for 5 min each. For detecting phosphorylated proteins, membranes were incubated with 1:10,000 dilution of PY-20

antiphosphotyrosine antibody in TBST for 1 h at room temperature. Membranes were washed five times with TBST for 5 min each. Chemiluminescence was then performed as described below. Membranes were also blotted with either 1:2000 dilution of a rabbit polyclonal antibody against IRS-laffinity purified on immobilized protein A or 1:1000 dilution of rabbit polyclonal antibody against IGF1R β (Santa Cruz Biotechnology, Inc.). Phospho-Akt (Ser⁴⁷³), Akt, phospho-p44/p42 ERK1/ERK2 (Thr²⁰²/Tyr²⁰⁴) and total ERK1/ERK2 antibodies were used as per manufacturer's instructions. Membranes were washed as before and incubated with 1:2000 dilution of antirabbit secondary antibody conjugated to HRP for 1 h at room temperature. Membranes were washed as before and chemiluminescence was done using Super-Signal West Pico substrate (Pierce, Rockford, IL).

Immunoprecipitations. A total of 500 μ g of total cellular proteins was first precleared with 25 μ l of protein A-agarose for 30 min and then incubated overnight with 3 μ l of polyclonal serum against IRS-1. A total of 25 μ l of protein A-agarose was added for 4 h. All steps were done on a rocker at 4°C. Immune complexes were collected by centrifugation at 12,000 \times g for 1 min. Immunoprecipitates were washed five times with 500- μ l each TNESEV by resuspension and centrifugation. After the final wash, immunoprecipitates were resuspended in 30 μ l of TNESEV and 30- μ l 2 \times Laemmli sample buffer containing 30 mg/ml DTT. Samples were boiled for 5 min, centrifuged, and supernatants were subjected to SDS-PAGE.

Proliferation Assays. Cells were plated in 24-well plates with 20,000 cells/well in regular growth medium. Cells were switched to SFM for 24 h and then treated as indicated in the figure legends. All treatments were done in triplicates. Growth was measured 4–5 days after treatment. Growth was assayed by the MTT assay as described previously (31). A total of 60 μ l of 5 mg/ml MTT solution in PBS was added to each well. After incubation for 4 h at 37°C, wells were aspirated and formazan crystals were lysed with 500 μ l of solubilization solution (95% DMSO + 5% IMEM). Absorbance was measured with a plate reader at 570 nm using a 670-nm differential filter.

Anchorage-independent Growth. Anchorage-independent growth assays were performed as described previously (28). A total of 1×10^4 cells/well of a 6-well plate was used. One ml of 0.8% SeaPlaque agarose (BioWhittaker, Rockland, ME) in culture medium was solidified in the bottom of each well as the bottom agar. Cells in growth media without or with 5 nM IGF-I, 250 nM scFv-Fc, or both IGF-I and scFv-Fc were mixed with 0.45% agarose. The cells mixed with agarose were overlaid on the bottom agar. After 12–14 days, colonies were counted using a light microscope with a grid in the eyepiece. The grid has 100 divisions and colonies larger than two-thirds of each square in the grid were counted. Three randomly selected fields were counted for each well, and the average number of colonies is shown. Results shown are representative of one experiment with triplicates for each treatment.

ADCC Assay. MCF-7 and SKBR-3 cells were labeled with ⁵¹Cr by incubating the cell pellets with 40 μ Ci of ⁵¹Cr for 1 h at 37°C. Human NK cells isolated from normal volunteer were used as effectors in the assay. Peripheral blood mononuclear cells were obtained from normal human blood by density gradient centrifugation using Ficoll-Paque. NK cells were isolated from peripheral blood mononuclear cell using a MACS NK cell isolation kit (Miltenyi Biotec, Auburn, CA) by depletion of non-NK cells according to the manufacturer's instructions. The purity of the NK cells was determined by flow cytometric analysis using CD56-PE and CD3-FITC and was >85% CD56⁺/CD3⁻ for all experiments. A total of 50,000 NK cells was mixed with 5000 ⁵¹Cr-labeled cells to give an effector:target ratio of 10:1 and incubated with various concentrations of either trastuzumab or scFv-Fc for 4 h. Each antibody dilution was tested in triplicate. The ability of the antibodies to cause cell lysis was measured by counting the released ⁵¹Cr. The results are shown as percentage of lysis plotted against the concentration of the antibodies.

Animal Studies and Xenograft Tumor Extract Analyses. Sixty-day release pellets of 17 β -estradiol (Innovative Research of America, Sarasota, FL) were implanted s.c. into 4-week-old female athymic mice. Each mouse was implanted with a 0.5-mg pellet. The next day, 5×10^6 MCF-7 cells in serum-free IMEM (without phenol-red) were injected into mammary fat pads on each side of each mouse. Tumor growth was measured weekly. When MCF-7 xenograft tumors were established (3 weeks), tumors from the left side of all mice were resected before treatment. Five mice were then injected i.p. with 500 μ g of scFv-Fc in PBS, and 5 mice were injected with PBS as control. Twenty-four h after injection of PBS or scFv-Fc, mice were sacrificed, and the remaining tumors were harvested. The tumors were snap frozen in liquid

nitrogen. Frozen tumor samples were homogenized in a tissue pulverizer in a dry ice/ethanol bath. Tissue homogenates were suspended in 500- μ l lysis buffer TNE5V. Homogenates were centrifuged at 12000 \times g for 20 min, and supernatants were stored at -70°C . One hundred μ g of each tumor extract along with 40 μ g of MCF-7 cell lysate were subjected to reducing SDS-PAGE on 8% acrylamide gels followed by immunoblotting for IGF1R β .

RESULTS

scFv-Fc Does Not Inhibit IGF-I Induced Phosphorylation of IRS-1 in MCF-7 Cells. Since the scFv-Fc has been shown to block IGF-I binding to IGF1R and partially inhibit xenograft growth of MCF-7 cells in mice, we first examined the effect of scFv-Fc on activation of IRS-1 in MCF-7 cells. In MCF-7 cells, we have previously shown that IRS-1 is the major adaptor protein phosphorylated by IGF-I-mediated activation of IGF1R. As reported previously (27) and shown in Fig. 1A, IGF-I treatment resulted in detection of a 185-kDa phosphorylated band in IGF-I-treated cells (Fig. 1A, Lane 2) but not in untreated cells (Fig. 1A, Lane 1). Pretreatment of cells with 250 nM scFv-Fc before stimulation with IGF-I did not inhibit IGF-I-induced phosphorylation of IRS-1 (Lane 3 compared with Lane 2) in Fig. 1A. Surprisingly, treatment with 250 nM scFv-Fc alone (Fig. 1A, Lane 4) resulted in phosphorylation of 185-kDa IRS-1 protein in a manner similar to IGF-I. As a control, cells were also treated with IGFBP-1. We have previously shown that IGFBP-1 blocks IGF-I action (28, 32) and as shown in Fig. 1A, Lane 5, pretreatment of cells with 8-fold molar excess of IGFBP-1 blocked IGF-I-mediated phosphorylation of IRS-1. IGFBP-1 treatment alone does not result in phosphorylation of IRS-1 (Fig. 1A, Lane 6). The lower panel shows that the total levels of IRS-1 were unchanged by treatment with scFv-Fc.

As both IRS-1 and IRS-2 have the same relative molecular mass, we wanted to confirm that the 185-kDa phosphorylated protein in cells treated with scFv-Fc was IRS-1. Therefore, cell lysates were immunoprecipitated with IRS-1 antibody followed by antiphosphotyrosine blotting (Fig. 1B). As shown in Fig. 1B, Lane 4, scFv-Fc treatment indeed resulted in phosphorylation of IRS-1 in MCF-7 cells similar to IGF-I (Fig. 1B, Lane 2). As a control in Fig. 1B, Lane 5, IGFBP-1 inhibited IGF-I-mediated phosphorylation of IRS-1 in MCF-7 cells.

scFv-Fc and IGF-I Activate Similar Downstream Signaling Pathways. We next determined whether scFv-Fc inhibited signaling pathways distal to IRS-1. We and others have shown that activation of

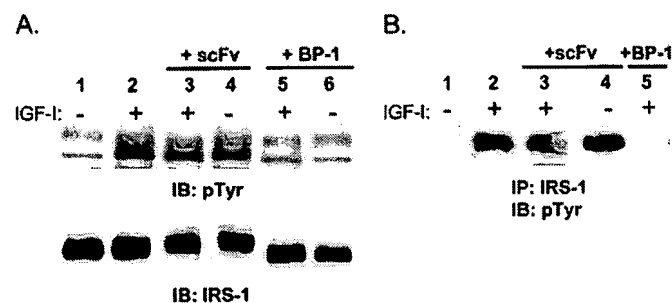


Fig. 1. scFv-Fc activates IRS-1 phosphorylation and does not block IGF-I effects. Seventy percent confluent MCF-7 cells were switched to SFM as described in "Materials and Methods." Twenty-four h later, cells were treated with 5 nM IGF-I or 250 nM scFv-Fc for 10 min. A, top panel, anti-phosphotyrosine immunoblot; bottom panel, IRS-1 immunoblot. Cells were either untreated (Lane 1), treated with 5 nM IGF-I (Lane 2), pretreated with scFv-Fc for 30 min prior to 5 nM IGF-I (Lane 3), or 250 nM scFv-Fc (Lane 4). As controls, in Lane 5 cells were pretreated with 40 nM IGFBP-1 for 30 min before stimulation with IGF-I, and in Lane 6, cells were treated with IGFBP-1 alone. B, immunoprecipitation with IRS-1 followed by immunoblotting with antiphosphotyrosine antibody. Lane 1 shows untreated cells, and Lane 2 shows cells treated with IGF-I. Lane 4 is cells treated with scFv-Fc alone.

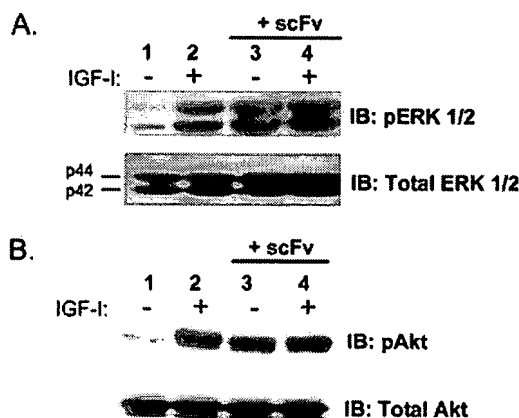


Fig. 2. scFv-Fc and IGF-I activate similar signaling pathways. A, activation of ERK1/ERK2 of the MAPK family. Cells were treated as described for Fig. 1 and then immunoblotted for activated ERK1/ERK2 (top panel) and total ERK1/ERK2 (bottom panel). Both the p42 and p44 kDa ERK1/ERK2 are shown. B, activation of Akt/PKB. Cellular proteins were immunoblotted for phosphorylated Akt (top panel) and total Akt (bottom panel). In both A and B, cells were untreated (Lane 1), treated with 5 nM IGF-I (Lane 2), or 250 nM scFv-Fc for 10 min (Lane 3). In Lane 4, cells were pretreated with scFv-Fc for 30 min before being stimulated with IGF-I for 10 min.

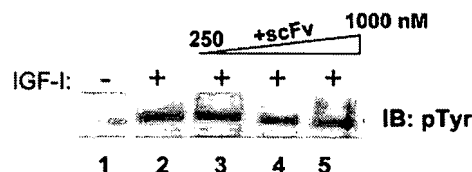


Fig. 3. scFv-Fc does not inhibit IGF-I-mediated effects, even at increasing concentrations. MCF-7 cells were pretreated with various concentrations of scFv-Fc for 30 min before stimulation with 5 nM IGF-I for 10 min. Cellular proteins were then immunoblotted for antiphosphotyrosine. Cells were untreated (Lane 1) or treated with 5 nM IGF-I for 10 min (Lane 2). Cells were also pretreated with 250 nM (Lane 3), 500 nM (Lane 4), or 1000 nM (Lane 5) scFv-Fc for 30 min before IGF-I treatment.

IGF1R by IGF-I results in phosphorylation of p44/p42 (ERK1/ERK2) of the MAPK pathway (27) and activation of the PI3K pathway (27, 33). We were interested in determining whether scFv-Fc perhaps inhibited IGF-I-induced activation of these downstream signaling pathways, which are linked to mitogenesis in MCF-7 cells. Thus, MCF-7 cells were treated with scFv-Fc before stimulation with or without IGF-I, and activation of ERK1/ERK2 was measured using a phosphospecific p44/p42 antibody. scFv-Fc did not inhibit IGF-I-induced phosphorylation of p44/p42 ERK1/ERK2 (Fig. 2A, Lane 4 versus Lane 2). Furthermore, scFv-Fc alone induced phosphorylation of ERK1/ERK2 (Fig. 2A, Lane 3). The time course of ERK1/ERK2 activation by scFv-Fc was similar to that by IGF-I (data not shown).

We then examined the PI3K pathway in MCF-7 cells. Measurement of phosphorylated Akt, a substrate for PI3K, was used to measure activation of this pathway. scFv-Fc did not inhibit IGF-I-mediated phosphorylation of Akt as shown in Fig. 2B (Lane 4). Similar to the case with ERK1/ERK2, scFv-Fc also resulted in phosphorylation of Akt as shown in Lane 3 of Fig. 2B. However, scFv-Fc caused a sustained phosphorylation of Akt compared with IGF-I (data not shown). Thus, biochemically, scFv-Fc activated IGF1R and downstream signaling pathways in MCF-7 cells *in vitro* in a manner similar to IGF-I.

To rule out the possibility that the inability of scFv-Fc to inhibit IGF-I induced phosphorylation of IRS-1 and activation of downstream signaling pathways was because of suboptimal concentrations of scFv-Fc, MCF-7 cells were pretreated with increasing concentrations of scFv-Fc from 250 to 1000 nM before stimulation with 5 nM

IGF-I. scFv-Fc at either 500 nM (as shown in Fig. 3, Lane 4) or 1000 nM (Fig. 3, Lane 5) did not inhibit IGF-I induced phosphorylation of IRS-1. This indicated that the inability of scFv-Fc to block IGF-I was not a concentration-dependent effect because even a 200-fold molar excess of scFv-Fc did not inhibit activation of IRS-1 in MCF-7 cells.

scFv-Fc Stimulates Activation of IGF1R and Downstream Signaling Molecules in Cells Overexpressing IGF1R. It has been reported that the inhibitory or stimulatory behavior of some antibodies may be dependent on cell surface receptor number (9, 34). To determine whether perhaps the stimulatory behavior scFv-Fc was dependent on the density of IGF1R in the cells, we used a NIH3T3 cell line that stably overexpresses IGF1R (NIH3T3/IGF1R). NIH3T3/IGF1R cells express ~1000-fold more receptors/cell compared with MCF-7 cells (25). As with MCF-7 cells, the ability of scFv-Fc to inhibit IGF-I mediated activation of IGF1R in these cells was studied by treating cells with scFv-Fc for 30 min before stimulation with IGF-I. In addition, NIH3T3/IGF1R cells were also treated with 250 nM scFv-Fc for 10 min to examine its effect on IGF1R in these cells. As these cells overexpress IGF1R, both phosphorylated IRS-1 (185 kDa) and phosphorylated IGF1R (97 kDa β -subunit) were seen on an antiphosphotyrosine immunoblot after IGF-I treatment (Fig. 4A, Lane 2). In NIH3T3/IGF1R cells, scFv-Fc did not inhibit IGF-I mediated activation of IGF1R as shown in Fig. 4A, Lane 3 or further downstream ERK1/ERK2 (Fig. 4C, Lane 3) or PI3K pathway (Fig. 4D, Lane 3). scFv-Fc alone also activated IGF1R (Fig. 4A, Lane 4), ERK1/ERK2 of the MAPK pathway (Fig. 4C, Lane 4), and Akt (Fig. 4D, Lane 4). Fig. 4B shows that the total levels of IRS-1 are unchanged by treatment with scFv-Fc (Lane 4).

scFv-Fc Causes Degradation of IRS-1. IGF-I causes ubiquitination followed by proteasomal mediated degradation of IRS-1 after 2 h, and levels of IRS-1 are greatly reduced by 8 h of IGF-I treatment (35). We assayed the effect of scFv-Fc treatment on levels of IRS-1. As shown in Fig. 5, scFv-Fc resulted in down-regulation of IRS-1 when examined by immunoblot, consistent with the effects of IGF-I.

scFv-Fc Stimulates *In Vitro* Proliferation of MCF-7 Cells. Because scFv-Fc behaved as a biochemical agonist of IGF1R in MCF-7



Fig. 5. scFv-Fc causes degradation of IRS-1. MCF-7 cells were left untreated (lanes labeled SFM) or treated with 5 nM IGF-I (lanes labeled IGF) or 250-nM scFv-Fc (lanes labeled scFv-Fc) for times as indicated in the figure. Cell lysates were then immunoblotted for total levels of IRS-1.

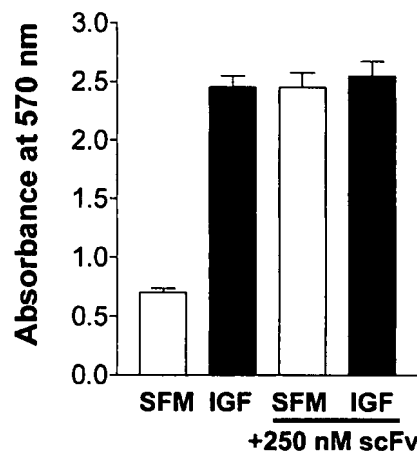


Fig. 6. scFv-Fc stimulates *in vitro* proliferation of MCF-7 cells. *In vitro* proliferation assay was performed using the MTT assay. MCF-7 cells in serum-free conditions were treated with IGF-I, scFv-Fc or IGF and scFv-Fc together for 5 days. Proliferation was then measured by the uptake of the MTT reagent followed by measurement of absorbance at 570 nm. Growth is shown as the absorbance at 570 nm and the data represent the mean \pm SE of four independent experiments with triplicate samples in each experiment.

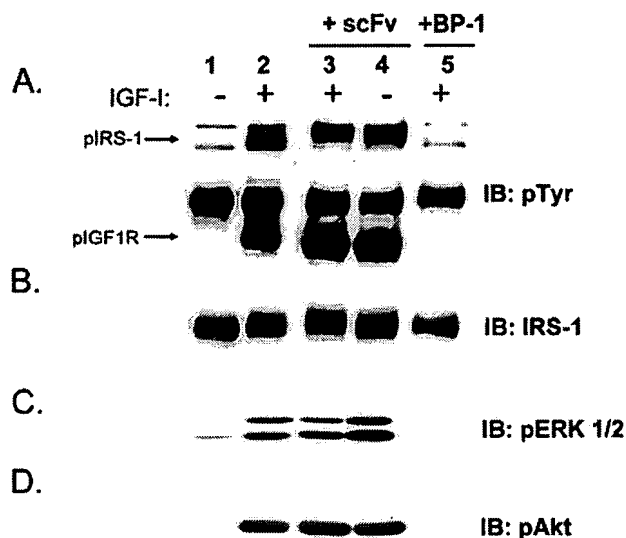


Fig. 4. Activation of IGF1R by scFv-Fc is not dependent on cell receptor number. NIH3T3 cells overexpressing IGF1R were treated as described in Fig. 1, and cell lysates were immunoblotted for phosphotyrosine (A), IRS-1 (B), phosphoERK1/ERK2 (C), and phosphoAkt (D). Cells were either untreated (Lane 1), treated with 5 nM IGF-I (Lane 2), pretreated with scFv-Fc for 30 min before 5 nM IGF-I (Lane 3), or treated with 250 nM scFv-Fc (Lane 4). In Lane 5, as a control to show that signaling pathways activated by IGF-I can be blocked, cells were pretreated with IGF1R-BP-1 for 30 min before stimulation with IGF-I.

cells, we next examined its effect on *in vitro* proliferation of MCF-7 cells. Not surprisingly, 250 nM scFv-Fc also stimulated the proliferation of MCF-7 cells to a similar extent as 5 nM IGF-I as shown in Fig. 6. This assay measures the ability of cells to proliferate for a short-term in serum-free conditions in response to growth factors or mitogens while attached to tissue culture plates. Although this assay measures a different phenotype than xenograft growth in mice, it nevertheless shows a discrepancy in the effect of scFv-Fc on IGF-I mediated mitogenic response of MCF-7 cells *in vitro* compared with *in vivo* growth in athymic mice.

scFv-Fc Stimulates Anchorage-independent Growth of MCF-7 Cells. To further explore the growth effects of scFv-Fc, we examined the ability of the antibody to inhibit IGF-I stimulated anchorage-independent growth. Perhaps the scFv-Fc could have a partial inhibitory effect on the xenograft growth of MCF-7 cells by affecting anchorage-independent growth. The results of the anchorage-independent growth assay in Fig. 7 are shown as the average number of colonies from triplicate wells. scFv-Fc did not inhibit IGF-I stimulated anchorage-independent growth of MCF-7 cells, but the antibody stimulated anchorage-independent growth, similar to the results in monolayer growth assays.

scFv-Fc Does Not Enhance ADCC. As scFv-Fc is a chimeric antibody with the Fc region of human IgG1 fused to the scFv, the results obtained *in vivo* could be because of activation of ADCC. Indeed, this mechanism of xenograft growth inhibition has been shown in trastuzumab-treated animals (36). NK cells have receptors for Fc, and this could be one of the mechanisms by which scFv-Fc partially inhibited tumor growth (37). Athymic mice, although deficient in T cells, have elevated levels of NK cells (38). We, therefore, performed an *in vitro* ^{51}Cr release cytotoxicity assay using MCF-7

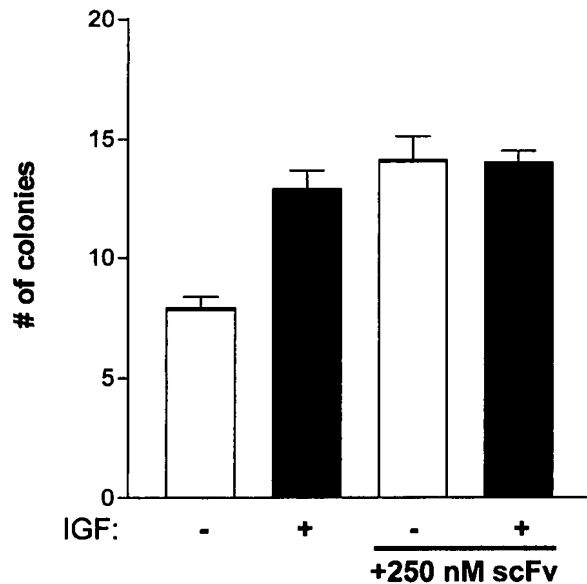


Fig. 7. scFv-Fc stimulates anchorage-independent growth. MCF-7 cells without or with 5 nM IGF-I or 250 nM scFv-Fc were mixed with 0.45% agarose and overlaid over 0.8% bottom agar in 6-well plates. Using a grid, colonies formed were counted on a portion of the well. Three randomly selected fields were counted for each well and averaged. Each treatment was done in triplicate, and the results are shown as the average number of colonies \pm SE. The experiment was repeated three times with similar results, and a representative experiment is shown.

cells as targets and NK cells purified from human peripheral blood mononuclear lymphocytes. As a positive control, we used trastuzumab along with SKBR3 cells, which overexpress HER2/c-erbB2. As shown in Fig. 8, scFv-Fc did not enhance lysis of MCF-7 cells or SKBR3 cells. The positive control trastuzumab caused increased lysis of both MCF-7 and SKBR3 targets.

scFv-Fc Causes Down-Regulation of IGF1R. In an attempt to determine the mechanism by which scFv-Fc could partially inhibit xenograft growth of MCF-7 in mice yet stimulate *in vitro* proliferation, we measured the levels of IGF1R after treatment of MCF-7 cells with scFv-Fc compared with IGF-I. Cells were treated with 5 nM IGF-I, 250 nM scFv-Fc, or both for different time periods. IGF1R levels were measured by immunoblotting with an antibody against the β -subunit of IGF1R. Untreated cells in Lane 1 (for each time point) showed no change in IGF1R levels from 10 min to 24 h as seen in Fig.

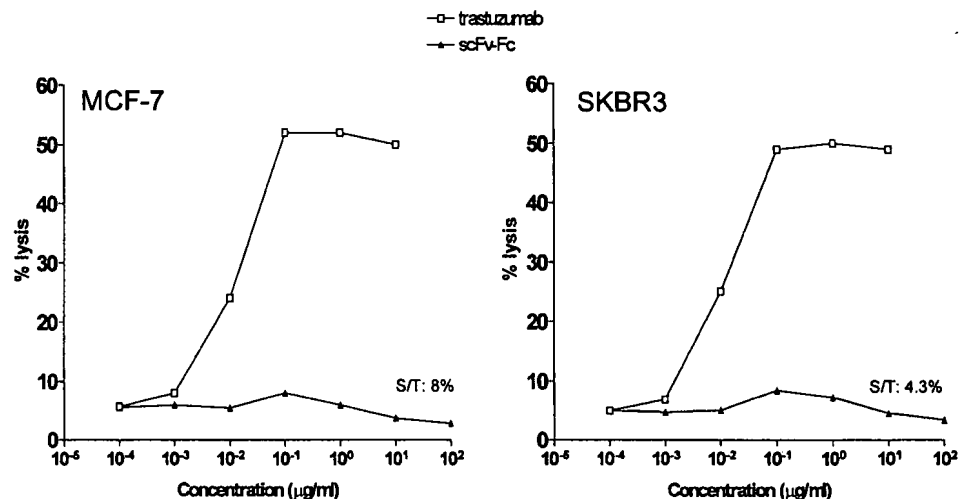
9A. Similarly, IGF-I treatment from 10 min to 24 h did not alter levels of IGF1R (Fig. 9A, Lane 2 compared with Lane 1). In contrast, scFv-Fc caused a rapid down-regulation of IGF1R after 2 h as shown in Fig. 9A, Lane 3, and by 24 h, very little IGF1R was detected. This effect of scFv-Fc in down-regulating IGF1R appeared to be a dominant effect as cells treated with IGF-I and scFv-Fc together (Fig. 9A, Lanes 4) also showed a marked down-regulation of IGF1R.

As many signaling molecules are regulated by ubiquitination followed by degradation by the proteasome, we investigated whether the mechanism by which scFv-Fc down-regulates IGF1R is via the proteasome pathway. We used the proteasome inhibitor MG115 (39) to block the proteolytic activity of the proteasomes. Cells were pretreated with 30 μ M MG115 for 2 h and then with IGF-I or scFv-Fc for 20 h. As shown in Fig. 9B, MG115 did not inhibit down-regulation of IGF1R by scFv-Fc (Lane 3; MG115 treatment) compared with cells treated with only scFv-Fc (Lane 3, no pretreatment). This suggests that down-regulation of IGF1R by scFv-Fc does not occur via the proteasomal pathway.

To determine whether down-regulation of IGF1R was mediated by the endocytic pathway, cells were pretreated with 40 mM methylamine (40) and then treated with IGF-I or scFv-Fc for various times. Methylamine raises pH of endosomes and inhibits the endocytic pathway. Pretreatment of cells with methylamine for 4 h blocked scFv-Fc-mediated down-regulation of IGF1R as seen in lanes marked 3 (for each time point) in Fig. 9C compared with cells that were not pretreated with methylamine (Lanes 3 in A). This suggests that down-regulation of IGF1R by the scFv-Fc occurs via the lysosomal/endocytic pathway as increasing the endosomal pH using the lysosomotropic agent methylamine inhibited receptor down-regulation.

Pretreatment of Cells with scFv-Fc Causes Inhibition of IGF-I Mediated Phosphorylation of IRS-1 and Activation of MAPK and PI3K Pathways. If the down-regulation of IGF1R by scFv-Fc was responsible for the *in vivo* growth inhibition of MCF-7 cells, we hypothesized that cells should not respond to additional IGF-I stimulation after antibody induced down-regulation of IGF1R. To determine whether this was the case, we pretreated cells with either IGF-I or scFv-Fc for 24 h. After this pretreatment, cells were left either untreated (lanes labeled SFM) or stimulated with additional 5 nM IGF-I. In Fig. 10A, Lanes 1–3 show that both IGF-I and scFv-Fc treatment resulted in phosphorylation of IRS-1 compared with untreated cells (Lane 1). When cells were pretreated with IGF-I for 24 h and then treated with additional 5 nM IGF-I for 10 min, they still

Fig. 8. scFv-Fc does not enhance ADCC. 51 Cr-labeled MCF-7 or SKBR3 cells were mixed with human NK cells at a ratio of 1:10 and incubated with various concentrations of scFv-Fc (\blacktriangle) or trastuzumab (\square) for 4 h. Cells were harvested, and cpm of 51 Cr released by lysis was counted using a γ -counter. Percentage of lysis of cells was calculated using $\text{Sp}/\text{T}-\text{S}$, where Sp is the specific lysis caused by antibody, S is the spontaneous lysis of cells in the absence of antibody, and T is the total lysis of cells by Triton X-100. Spontaneous lysis of cells was $<8\%$. The results are plotted as percentage of lysis against the concentration of antibody. The data shown is representative of three experiments with similar results.



Effector:Target 10:1

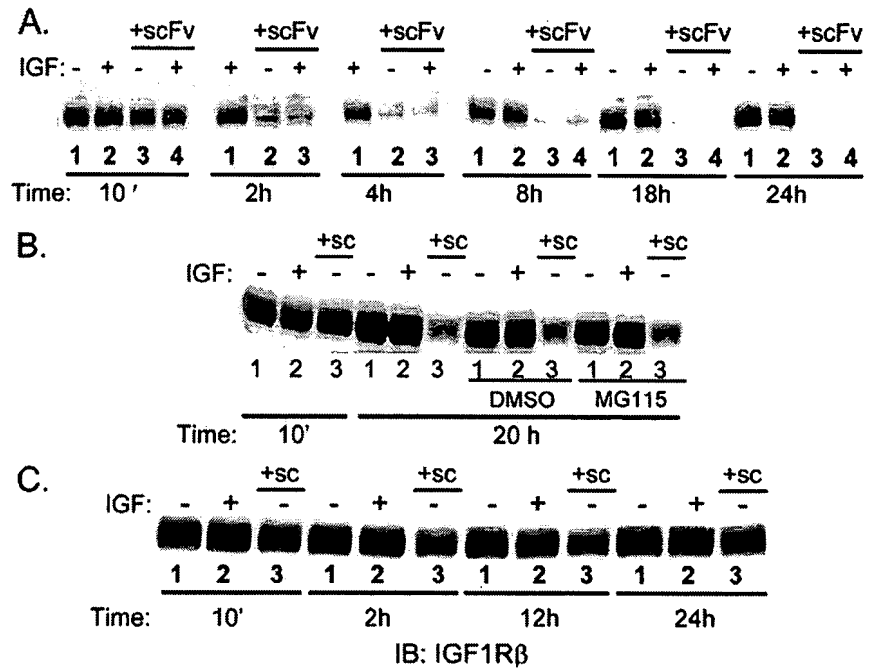


Fig. 9. scFv-Fc causes rapid down-regulation of IGF1R via the endocytic pathway. MCF-7 cells were left untreated (lanes labeled 1) or treated with 5 nM IGF-I (Lanes 2), 250 nM scFv-Fc (Lanes 3), or IGF-I + scFv-Fc (Lanes 4) for various times as indicated below the lane number. In B, cells were pretreated with 30 μ M MG115 in DMSO or DMSO alone for 2 h and then treated with IGF-I or scFv-Fc for 20 h. In C, cells were pretreated with 40 mM methylamine for 4 h before treatment with IGF-I or scFv-Fc for various times. Cell lysates were then immunoblotted for total levels of IGF1R β . The M_r 97,000 β -subunit of IGF1R is shown in the figure.

retained the ability to phosphorylate IRS-1 (Fig. 10A, Lane 5 compared with Lane 4). In contrast, when cells were pretreated with scFv-Fc for 24 h followed by treatment with 5 nM IGF-I for 10 min, phosphorylated IRS-1 was not detected (Fig. 10A, Lane 7). Cells pretreated with scFv-Fc then re-treated with additional antibody did not have enhanced IRS-1 phosphorylation (data not shown). Further-

more, as shown in Fig. 10, pretreatment of cells with scFv-Fc for 24 h before treatment with IGF-I for 10 min also blocked subsequent phosphorylation of p44/p42 ERK1/ERK2 (Fig. 10B, Lane 7) and Akt (Fig. 10C, Lane 7 compared with Lane 6). In contrast, pretreatment with IGF-I for 24 h does not inhibit stimulation of these pathways. Additional IGF-I still leads to activation of both the ERK1/ERK2 (Fig. 10B, Lane 5) and PI3K pathways (Fig. 10C, Lane 5).

Thus, 24-h pretreatment of cells with scFv-Fc inhibited the ability of 5 nM IGF-I to phosphorylate IRS-1 and blocked subsequent ERK1/ERK2 and PI3K activation. In contrast, cells treated with 5 nM IGF-I for 24 h still retained the ability to be additionally stimulated by subsequent treatment with additional IGF-I. These data show that down-regulation of IGF1R by scFv-Fc blocked the ability of IGF-I to initiate mitogenic signaling.

Pretreatment of Cells with scFv-Fc Makes Them Refractory to Additional IGF-I Mediated Proliferation. Since scFv-Fc caused down-regulation of IGF1R and inhibition of IGF-I-mediated activation of ERK1/ERK2 and PI3K pathways, we tested if 24-h pretreatment of cells with scFv-Fc before exposure to additional IGF-I would have any effect on the growth of cells. As shown in Fig. 5, scFv-Fc stimulated MCF-7 cells to proliferate. Here, we were interested in studying the further response of cells to additional IGF-I after 24-h pretreatment with scFv-Fc. Cells were pretreated with IGF-I or scFv-Fc for 24 h. After this pretreatment, cells were washed and incubated with or without additional IGF-I. Cell growth was measured after 4 days. Pretreatment with IGF-I and treatment with subsequent IGF-I resulted in increased proliferation compared with the control as shown in Fig. 11. In contrast, cells pretreated with scFv-Fc did not proliferate further in response to additional IGF-I.

IGF1R down-regulation by scFv-Fc also inhibited anchorage-independent growth of MCF-7 cells. As shown in Fig. 12, cells pretreated with IGF-I for 24 h had increased colony formation in soft agar in response to IGF-I. In contrast, cells pretreated with scFv-Fc had decreased colony formation.

Thus, the difference between these results and that in Figs. 6 and 7 relates to whether or not IGF1R was present on the cell surface when the cells were exposed to additional IGF-I. Although scFv-Fc activates receptor initially, when cells are re-treated with IGF-I, the

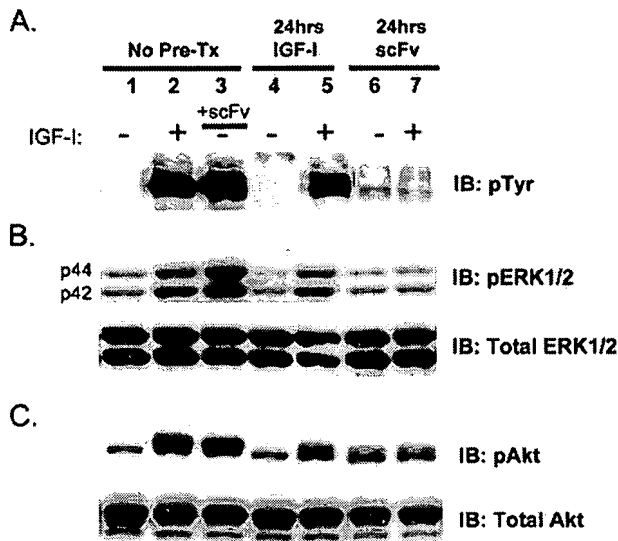


Fig. 10. Pretreatment of cells with scFv-Fc inhibits phosphorylation of IRS-1 and activation of downstream signaling pathways induced by IGF-I. A, antiphosphotyrosine immunoblot. B, phospho-ERK1/ERK2 immunoblot. C, phospho-Akt immunoblot. Cells were pretreated with either IGF-I (lanes labeled 24 h IGF) or scFv-Fc (lanes labeled 24 h scFv) for 24 h. After this pretreatment, cells were either left untreated (lanes labeled -IGF-I) or stimulated with 5 nM IGF-I (lanes labeled +IGF-I). Lanes 1-3 show that both IGF-I and scFv-Fc phosphorylate IRS-1 compared with untreated cells (Lane 1). Lanes 4 and 5 show cells pretreated with IGF-I for 24 h and then treated with an additional 5 nM IGF-I for 10 min (Lane 5) or untreated (Lane 4). Lanes 6 and 7 show cells pretreated with scFv-Fc for 24 h and then treated with 5 nM IGF-I for 10 min (Lane 7) or untreated (Lane 6). When cells are pretreated with scFv-Fc for 24 h followed by treatment with 5 nM IGF-I for 10 min, they no longer phosphorylate IRS-1 (Lane 7 compared with Lane 2). Furthermore, pretreatment of cells with scFv-Fc for 24 h also blocked subsequent phosphorylation of p44/p42 ERK1/ERK2 (B, Lane 7) and phosphorylation of Akt (C, Lane 7 compared with Lane 6) by IGF-I.

scFv-Fc treated cells do not respond, whereas the IGF-I treated cells still have ample receptor on the cell surface allowing for additional increase in proliferation.

scFv-Fc Causes Down-Regulation of IGF1R *in Vivo* in Xenograft Tumors in Mice Injected with the Antibody. To confirm if our *in vitro* findings of down-regulation of IGF1R levels by scFv-Fc was responsible for inhibition of tumor growth *in vivo*, we tested the effect of scFv-Fc on IGF1R levels in mice with MCF-7 xenograft tumors. Ten mice bearing two xenograft tumors each, one on either side, were studied. Tumors were resected from the left side of all 10

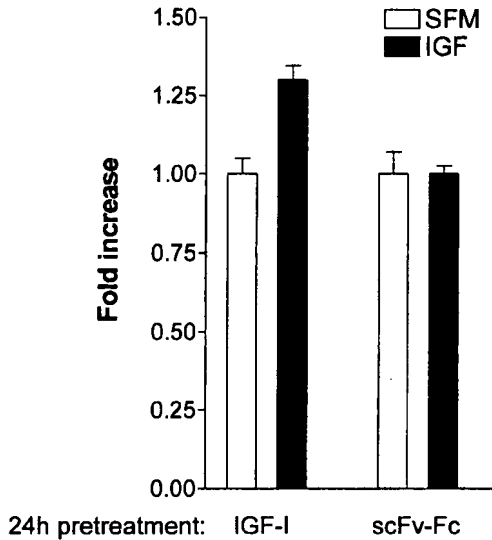


Fig. 11. Pretreatment of cells with scFv-Fc renders them refractory to additional growth stimulation by IGF-I. *In vitro* proliferation assay was performed using the MTT assay. MCF-7 cells in serum-free conditions were pretreated with either IGF-I (bars labeled 24 h pretreatment with IGF) or scFv-Fc (bars labeled 24 h pretreatment with scFv) for 24 h. Media were removed, and cells were washed and then treated without (SFM) or with 5 nM IGF-I (IGF-I) for 5 days. Proliferation was then measured by the uptake of the MTT reagent followed by measurement of absorbance at 570 nm. Growth is shown as the fold increase over control and the data represent the mean \pm SE of triplicate readings from two experiments.

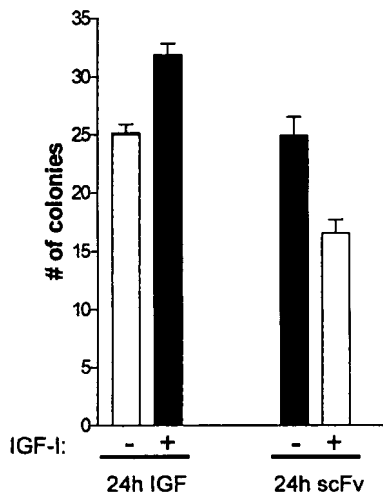


Fig. 12. Pretreatment of cells with scFv-Fc inhibits IGF-I-stimulated colony formation in soft agar. Cells were pretreated with scFv-Fc or IGF-I for 24 h. MCF-7 cells were then washed, trypsinized, centrifuged, and resuspended in medium. Cells without or with 5 nM IGF-I were mixed with 0.45% agarose and overlaid over 0.8% bottom agar in 6-well plates. Colonies formed were counted after 12 days using a microscope with a grid. Three randomly selected fields were counted for each well and averaged. Each treatment was done in triplicate, and the results are shown as the average number of colonies \pm SE. Similar results were obtained when the experiment was repeated twice, and a representative experiment is shown.

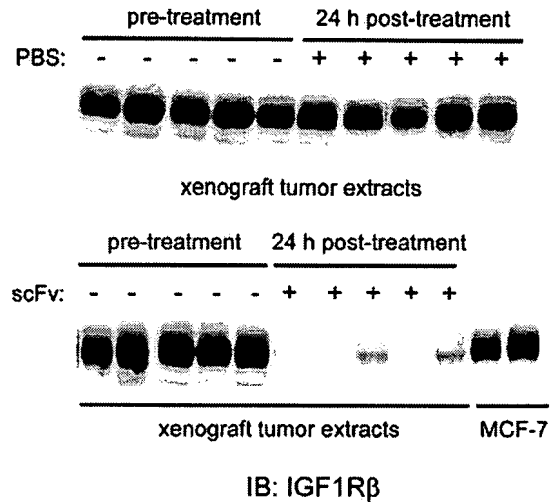


Fig. 13. scFv-Fc down-regulates IGF1R *in vivo*. Female athymic mice bearing MCF-7 xenograft tumors on each side were studied. Tumors from the left side were resected from each mouse before treatment. The next day, 5 mice received i.p. injections of 500 μ g of scFv-Fc, and 5 mice received i.p. injections of PBS. Twenty-four h after treatment, tumors were collected. Tumor samples were frozen in liquid nitrogen and homogenized in TNESV buffer using a pulverizer as described in "Materials and Methods." A total of 100 μ g of tumor extracts and 40 μ g of MCF-7 cell lysate (bottom panel; lanes labeled MCF-7) were immunoblotted for total IGF1R β levels. Total IGF1R levels in the tumor extracts in the 10 mice before treatment is shown in lanes labeled pre-treatment in the top and bottom panels. IGF1R levels in the mice that were injected with scFv-Fc is shown in lanes labeled 24 h post-treatment in the bottom panel. Tumor extracts from mice after treatment with PBS is shown in the top panel.

mice before treatment. The next day, 5 mice received i.p. injections of 500 μ g of scFv-Fc, and 5 mice received i.p. injections of control PBS. To allow sufficient time for scFv-Fc to cross the peritoneum, 24 h after treatment, mice were sacrificed and tumors harvested. All tumor extracts were then subjected to immunoblotting for IGF1R. Fig. 13 shows that all MCF-7 xenograft tumors had high levels of IGF1R before treatment (lanes labeled pre-treatment in the top and bottom panel). Twenty-four h after treatment, tumors taken from animals treated with scFv-Fc had significant down-regulation of IGF1R (bottom panel, lanes labeled 24 h post-treatment). No change in IGF1R was observed in PBS-treated animals (top panel). Densitometric analysis showed that after treatment with scFv-Fc, IGF1R levels in xenograft tumor extracts were \sim 22% ($n = 5$) of that in tumor extracts before scFv-Fc treatment. In contrast, in mice that received control PBS injections, the IGF1R levels were \sim 99% of that before treatment.

DISCUSSION

The IGF system had been implicated in various roles in carcinogenesis. A truncated dominant negative IGF1R has been reported to inhibit the metastasis of MDA-MB-435 breast cancer cells (41, 42). In breast cancer patients, tumors with high IGF1R levels have increased ipsilateral breast recurrence of tumor after lumpectomy and radiation (5). Antisense IGF1R strategies are being tested in astrocytomas and neuroblastomas (43).

Thus, IGF1R is being tested as a target for cancer therapeutics. An obvious strategy to disrupt IGF-I action would be to develop small molecule inhibitors of the tyrosine kinase activity of IGF1R. Whereas small molecule inhibitors such as Iressa (ZD1839) have been successfully used to inhibit the tyrosine kinase activity of EGFR (44, 45) and also HER2, it has been difficult to do the same with IGF1R. IGF1R shares a high degree of homology to the insulin receptor (46), making it difficult to design small molecule tyrosine kinase inhibitors specific for IGF1R. Although some such inhibitors have been described, they all have the ability to inhibit the insulin receptor (17). The determi-

nation of higher resolution crystal structures of the tyrosine kinase domain of IGF1R (47) may enhance the design of a tyrosine kinase inhibitor that is highly specific for IGF1R.

However, antibodies that can be recombinantly engineered to specifically target growth factor receptors are also a very attractive option. The success of trastuzumab, a humanized monoclonal antibody against the extracellular domain of HER2/c-erbB2 receptor, in the treatment of breast cancer patients whose tumors overexpress HER2 supports this approach. A chimeric antibody developed against EGFR is currently in clinical trial (48). Here, we have examined a chimeric antibody against IGF1R that was engineered from an inhibitory monoclonal antibody, 1H7, that blocks binding of IGF-I and IGF-II to IGF1R (26). scFv-Fc was engineered by PCR and is a chimeric antibody that has the Fc domain of human IgG1 fused to the Fv region of the mouse monoclonal antibody 1H7.

We show here that scFv-Fc behaves as a biochemical agonist of IGF1R. It activates IGF1R and downstream signaling pathways in a fashion identical to IGF-I. There are other antibodies against IGF1R that also enhance receptor autophosphorylation (49). In the case of monoclonal antibodies against HER2/erbB2, several have been described that phosphorylate HER2 yet inhibit growth (50). Thus, there is no clear correlation between biochemical activation of receptor by an antibody and growth inhibitory properties (51). Surprisingly, scFv-Fc also stimulated *in vitro* growth of MCF-7 cells in short-term cultures. This was contrary to our expectation as we undertook these studies to understand the mechanism by which scFv-Fc inhibits *in vivo* growth of MCF-7 cells. Our *in vitro* monolayer and anchorage-independent growth experiments suggest that only a brief exposure of a stimulating ligand will result in apparent proliferation in these short-term assays. It is possible that activation of IGF1R signaling pathways, even for only a few hours, results in increased cell number when measured 5–7 days later. Thus, *in vitro* growth assays may not always be a good prediction of effects *in vivo*. Although the exact mechanism by which antibodies inhibit tumor growth is not understood, there is evidence that the *in vitro* and *in vivo* effects of antibodies can be opposing as has been reported for antibodies against ErbB2 (52). This may be because the *in vivo* effects of an antibody are also dependent on host-tumor interactions, and the tumor microenvironment also has an impact.

As scFv-Fc is a chimeric antibody and is partially humanized with the Fc region of human IgG1, it is possible that one of the mechanisms by which scFv-Fc inhibits xenograft growth of MCF-7 cells *in vivo* is by enhancing ADCC. However, our *in vitro* data suggest that ADCC is not the major mechanism by which scFv-Fc caused inhibition of tumor growth. Unlike trastuzumab, scFv-Fc did not enhance lysis of MCF-7 cells in an *in vitro* ADCC assay.

A second mechanism by which scFv-Fc could inhibit tumor growth is by its effect on IGF1R levels. scFv-Fc caused down-regulation of IGF1R both *in vitro* and in athymic mice with xenograft tumors that received 500 μ g of scFv-Fc. The mechanism by which this occurs is not yet clear. The down-regulation of IGF1R does not occur via the proteasome-ubiquitin pathway as the proteasome inhibitor MG115 had no effect on scFv-Fc induced receptor down-regulation. In contrast, the lysomotrophic agent methylamine blocked scFv-Fc induced down-regulation of IGF1R implicating the endosomal endocytic pathway. IGF-I treatment did not cause changes in steady state levels of IGF1R, although IGF-I also causes endocytosis of its receptor (53, 54). It is possible that IGF-I induced receptor endocytosis is balanced by receptor recycling to the membrane (55). In contrast, perhaps scFv-Fc causes increased endocytosis but may not allow for receptor recycling, causing a net decrease in cell surface receptor levels over time. Such an increase in receptor turnover leading to down-modulation of the oncogene product of HER2/c-erbB2 has been reported with a monoclonal antibody against the receptor (56). It does not appear

that the tyrosine kinase activity of the receptor is required for IGF1R down-regulation because scFv-Fc also caused down-regulation of IGF1R in a metastatic variant of MDA-MB-435 cells, LCC6 cells (57), stably transfected with a truncated dominant negative IGF1R (data not shown). The antibody α IR3, which does not cause IGF1R tyrosine phosphorylation, also caused IGF1R down-regulation (data not shown). Although tyrosine kinase activation of the receptor is required for internalization of receptor by many monoclonal antibodies, it appears that tyrosine kinase activity is not always required for antibody-mediated down-regulation of IGF1R. It has been reported that tyrosine kinase activity is not required for internalization of a murine monoclonal antibody TA1 against HER2 (58).

It is possible that serine or threonine phosphorylation of IGF1R by scFv-Fc could contribute to IGF1R down-regulation. Xu *et al.* (50) have reported that a monoclonal antibody against HER2 that induces receptor down-regulation causes tyrosine phosphorylation of the receptor during short-term treatment and then causes serine phosphorylation of some intracellular substrates. It may also be that the scFv-Fc simply causes receptor aggregation leading to down-regulation.

Thus, *in vivo* repeated injection of scFv-Fc may cause sustained down-regulation of IGF1R, which results in inhibition of MCF-7 xenograft growth in mice. Using another mouse model of breast cancer, the T61 xenograft tumor model in athymic mice (59, 60), we have shown that i.p. injections of scFv-Fc resulted in decreased IGF1R levels in these tumors.⁴

A third mechanism may be that scFv-Fc alters the distribution of cell cycle components. It has been reported that cells that respond to IGF-I by a mitogenic response activate certain key cell cycle components (61). scFv-Fc may cause a decrease in the percentage of cells in the S phase, perhaps by induction of CDK2 kinase inhibitor p27^{KIP1}.

Given the fact that IGFs have pleiotropic effects on breast cancer, inhibition of IGF1R activation can be beneficial to breast cancer patients in several ways. Proliferation, cell metastasis, protection from chemotherapy, and radiation are all mediated by IGF1R. With recent reports indicating that activation of the IGF1R may be one of the mechanisms by which tumors become resistant to trastuzumab (62, 63), it may be even more important to target IGF1R as a potential breast cancer therapy. Our data show that down-regulation of IGF1R by a chimeric antibody could be a successful therapeutic strategy.

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